Strategies for Folate Biofortification in Soybean

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Folate (vitamin B9) is an essential water-soluble vitamin in plants and microorganisms that plays a role in one-carbon metabolism. It functions as a cofactor in the synthesis of nucleic acids, metabolism of amino acids, and methylation of hormones, lipids, proteins, and chlorophyll. Folate is particularly important for cell division in pregnant and lactating women. However, humans cannot synthesise folate de novo and must obtain it from dietary sources, such as crops, animal-based foods, or nutritional supplements.

Keywords: soybean ; breeding ; folate ; strategy ; biofortification

1. Structure, Distribution, Content and Composition of Folate in Soybean

1.1. Structure and Distribution of Folate Vitamers in Soybean

Folate comprises a pterin moiety attached by a methylene bridge to para-aminobenzoic acid coupled to one or more glutamyl residues. In vivo, folates exist as tetrahydrofolate (H_4 folate) and its derivatives, which vary in the state of oxidation of the pteridine ring, single carbon substituents linked at the 5 and 10 positions, and with a variable number of glutamyl residues ^[1]. In theory, over 150 folate derivatives exist, but a few exist in plants and humans.

Recent chromatographic methods have indicated the folate vitamers present in soybean seeds include H₄folate (THF), 5-CH₃-H₄folate (5MTHF), 5-CHO-H₄folate (5FTHF), H₂folate (DHF), 10-CHO-H₄folate, PteGlu (FA), 5,10-CH=H₄folate (5,10-MTHF), 10-CHO-PteGlu (10FFA), and MeFox (**Figure 1**). However, the distribution of these vitamers in soybean seeds has not been frequently reported. Soybean vitamer distribution can be influenced by factors such as storage, plant developmental stage, cultivar, accession type, environment and analytical method. Some studies have reported THF as the most abundant folate vitamer in soybean ^{[2][3]}, while other studies have identified 5FTHF as the most abundant ^{[4][5]}. 5FTHF accounted for more than 60% of the total folate content of soybean accessions grown in southern China ^[6]. Climate conditions, particularly temperature and humidity, may influence folate synthesis and accumulation. For example, lower temperatures have been shown to increase 5MTHF accumulation in sweet corn seedlings ^[2], while higher temperatures can lead to a significant decrease in the folate content of lettuce ^[8].



Figure 1. Chemical structures of folate vitamers in soybean: (A) H_4 folate (THF); (B) 5-CH₃-H₄ folate (5MTHF); (C) 5-CHO-H₄ folate (5FTHF); (D) PteGlu (FA); (E) H_2 folate (DHF); (F) 5,10-CH=H₄ folate (5,10-MTHF); (G) 10-CHO-H₄ folate; (H) 10-CHO-PteGlu (10FFA); (I) MeFox.

Whereas a few studies have reported THF as the most dominant vitamer in soybean ^{[2][3]}, that was not observed in researcher's recent studies ^[6]. THF is one of the most labile vitamers that can easily degrade or convert right from harvest. Researchers assume that THF might have been converted into FA or degraded right after harvest and during post-harvest storage. Studies on the homeostasis and dynamics of plant folates during storage have been limited. Biosynthesis may occur during post-harvest storage in some organisms, while variations in the folate pool in other organisms may be due to spontaneous or enzymatic reactions ^[9]. Further studies will be necessary to confirm vitamer distribution during post-harvest storage in soybean seeds.

1.2. The Folate Content and Composition of Soybean and Soy-Based Products

Soybean can be processed into various forms through germination, soaking, boiling, and fermentation $^{[10][11]}$. However, folates are sensitive to light, air, heat and pH; therefore, their content and composition may be indirectly affected during processing. Mature unprocessed soybean seeds contain total folate levels ranging from 64.51–691.24 µg/100 g FW and 199 to 464 µg/100 g DW (**Table 1**). The differences in folate contents can be attributed to cultivar type, environment, and analytical methods used. In researcher's recent study, researchers identified a 10-fold variation in the total folate content of over 1000 germplasm consisting of landraces and cultivars ^[6]. However, most studies have been limited to a few cultivars. A larger sample size is needed to evaluate the variation among soybean folates and, most importantly, different accession types (wild-type, landraces and cultivars).

Cooked soybean seeds contained lower levels of folate (44.70–77.90 μ g/100 g) at a retention rate of 24–45%, depending on the cooking treatment ^[12]. Similarly, significant losses were observed during the preparation of soymilk and tofu ^[11]. The significant loss of folates during tofu and soymilk processing was mainly caused by soaking and boiling, as most folates were recovered in the cooking or soaking media. Thus, the major cause of folate loss is leaching and, to a certain extent, oxidation. To avoid such losses, shorter boiling times and possible consumption of the cooking media are recommended.

Folate in tempeh ranged from 149.30 to 416.40 μ g/100 g. Fermentation contributes to the increase in folate during tempeh preparation. During fermentation, folate compounds may be liberated by the actions of enzymes produced by the microorganism, leading to increased folate concentrations. The de novo formation of folate compounds during fermentation may also increase folate content ^[5]. However, the extent of the increase of folates will depend on the microorganism involved ^[10].

Seed germination is an age-old practice used to improve the nutritional value of crops, especially legumes ^[13]. Germination affects folate profiles and alters the distribution of individual vitamers ^{[14][15]}. In soybean, folate content increased 3.5 to 3.7-fold from 230.50 to 815.20 μ g/100 g in Bangladesh soybean-4 and from 202.90–759.50 μ g/100 g in Heinong 48 ^[2]. In germinated soybean, approximately 80% of the total folate content is 5MTHF, the most active folate vitamer.

In researcher's recent study, the total folate content of immature soybean seeds at the R6–R7 stage, a typical stage for *edamame* or *maodou*, ranged between 344.06–685.81 μ g/100 g FW among 12 soybean cultivars, with 5MTHF contributing approximately 70% of the total folate content and 5FTHF contributing approximately 15% ^[16].

Sample	THF	5MTHF	5,10- MTHF	10FFA	5FTHF	DHF	FA	Total Folate	References
Soybean seed		16.90		53.80	121.00			199.00- 464.00	[4][5][11][17] [18]
Soybean seed	20.00– 75.00 *	28.00- 205.74 *	5.00- 28.60	11.00- 71.06	160.00- 590.56	2.90– 29.44	28.50– 34.40 *	64.51- 691.24 *	[2][3][6][12]
Soybean seed (cooked)								44.70– 77.90 *	[12]
Vegetable soybean	12.55 *	356.18 *	10.17 *	4.33 *	75.07 *	2.98 *	1.00 *	344.06- 685.81	[<u>16]</u>
Soymilk								34.00- 276.00	[<u>17][19]</u>
Tofu								15.00- 127.30	[10][17][19]

Table 1. The folate content of soybean seeds and soy-based products.

Sample	THF	5MTHF	5,10- MTHF	10FFA	5FTHF	DHF	FA	Total Folate	References
Tempeh					231.80			149.30- 416.40	[10][17]
Soybean sprouts								759.50– 815.20 *	[2]

The folate content of soybean seeds and soy-based products $\mu g/100$ g on dry weight basis. Values with * indicate folate content on fresh weight basis.

2. Prospects for Biofortification of Folates in Legumes

Biofortification can be classified based on a variety of approaches, including agronomic and genetic methods and the consumption of functional foods, such as fermented foods and sprouts ^{[20][21]}. Agronomic biofortification involves using agricultural techniques to increase the nutrient content in crops. This can include applying fertilizers, such as zinc or iron, or soil microbes to the soil in which the crops are grown ^{[22][23][24][25]}. The soil is the primary source of nutrients, and the abundance of nutrients in the soil and their availability to plants determine the synthesis of plant metabolites ^[26]. Agronomic biofortification is relatively simple and straightforward but can be expensive and time-consuming ^[27]. This is because this approach can be limited by the inherent variability in the soil nutrient availability, which is affected by factors such as soil type, climate and crop requirements. As a result, the effectiveness of agronomic biofortification may vary and may require extensive maintenance and input. To date, agronomic fortification has not been studied for folates in soybean. However, research has shown that the folate content of plants can increase when they have access to nutrients, such as phosphorus, nitrogen, and boron, suggesting that agronomic fortification of folates in soybean may be worth investigating in the future ^[28].

Genetic biofortification is the most well-known form of biofortification and has been widely studied and researched by scientists and plant breeders. It involves using genetic techniques to increase the nutrients in crops and can be achieved through several approaches, including conventional breeding, genomics-assisted breeding, and metabolic engineering.

Conventional breeding involves using traditional breeding techniques, such as crossbreeding and selection, to develop new varieties of crops that are rich in nutrients such as folate. Conventional breeding typically involves several steps, including identifying genetic variation in a germplasm pool, selecting elite-folate accessions and crossing these accessions with local varieties to create new varieties with improved nutrient content. One of the main advantages of conventional breeding is that it is a relatively low-cost and low-risk approach to biofortification. However, it can be a slow process, as it can take several years to develop and test new varieties of crops. Additionally, crops with low genetic variation for folate ^[9] may not be suitable for conventional breeding as there may not be sufficient genetic diversity to create new varieties with improved folate content.

Overall, conventional breeding can be an effective approach to folate biofortification, particularly in crops that have a high level of genetic variation, such as rice, potato and soybean ^{[6][29][30]}. Additionally, wild and landrace accessions of crops are often adapted to local growing conditions and may contain important genes that can be used to improve the nutrient content of cultivated species ^{[31][32]}. For example, wild lentils and soybean landraces have been found to contain higher folate content than cultivated accessions and introgressing these traits into cultivated varieties could help to increase the overall folate content of these crops ^{[6][33]}.

Genomics-assisted breeding involves the use of genomic tools and technologies such as marker-assisted breeding (MAB), marker-assisted selection (MAS), marker-assisted backcrossing (MABC) and marker-assisted recurrent selection (MARC) to improve crop breeding programs ^[34] (**Figure 2**). These techniques can be used in conjunction with multi-omics data, databases, and genes generated by genomics to better understand the genetic basis of traits such as folate and to develop new varieties with improved folate profiles. High-throughput genotyping methods, such as genotyping by sequencing (GBS), can be used to genotype a large number of accessions ^[35], while phenotyping methods can be used to measure the folate content of these accessions. Linkage mapping, genome-wide association studies (GWAS) ^[36], and bulked segregant analysis-sequencing (BSA-Seq) ^[37] can then be used to identify quantitative trait loci (QTL) ^[38] or quantitative trait nucleotides (QTN) ^[39] that are associated with folate content. RNA-Seq can be combined with identified QTL or QTN and analysed using weighted genes ^[40]. After the identification of candidate genes, functional analysis and metabolic pathway analysis can be conducted to further understand their roles in folate biosynthesis. MAB can then be utilised to develop new hybrids with improved folate content.



Figure 2. Framework for the genetic biofortification of folates in soybean. To improve the folate content of soybean, accessions can be genotyped using sequencing techniques, such as genotyping by sequencing (GBS) and phenotyped for their folate content. Linkage mapping, genome-wide association study (GWAS), and bulked-segregant analysis-sequencing (BSA-Seq) can be used to identify quantitative trait loci or quantitative trait nucleotides (QTL/QTN). The results from RNA-Seq can then be combined with QTL/QTN using weighted gene co-expression analysis (WGCNA) to identify candidate genes. These candidate genes can be further analysed for their functions and roles in the metabolic pathway. Finally, marker-assisted breeding (MAB) can be used to develop hybrids with improved folate content.

Many QTL and genes associated with folate content have been identified in various crops using these techniques, including pea ^[41], common bean ^{[42][43]}, rice ^[29], maize ^[44], potato ^[45] and sweet corn ^[46]. For example, a genome-wide association study in 85 pea accessions revealed 9 SNPs significantly associated with 5MTHF, 5FTHF, THF and total folate, with two SNPs linked to higher levels of 5MTHF and total folate content. Linkage mapping has also been used to identify 4 QTL for 5MTHF in common bean, while a GWAS in 96 common bean genotypes identified 6 QTL for folate accumulation, including QTL on chromosome 11 that occurred in genomic regions syntenic to already reported QTL in maize, rice and common bean ^[42] (**Table 2**). However, to date, there have been no studies on QTL/QTN related to folate components in soybean.

Сгор	Population Type	Population Size	Total Number of QTL Identified	PVE (%)	Model of Analysis	Reference
Pea	Natural population	85	9	-	MLM	[41]
Common bean	Bi-parental	6	4	8–19	SMA	[43]
Common bean	Natural population	96	6	-	Fast-LMM/EMMA	[42]

Table 2. QTL identified for folate content in legumes.

PVE—phenotypic variation explained; MLM—mixed linear model; SMA—single marker analysis; Fast-LMM—factored spectrally transformed linear mixed model; EMMA—efficient mixed model analysis.

One way to improve the folate content of crops is through metabolic engineering. This involves modifying the genetic makeup of crops to increase the production of specific nutrients or other bioactive compounds. Conventional breeding techniques may not always be sufficient to improve the folate content of crops in which case metabolic engineering can be used ^[21]. The metabolic engineering of folates has focused on enhancing folate synthesis ^[20]. Most of the enzymes that partake in the biosynthesis of folates have been characterised and cloned. However, transcriptomic and gene expression studies have revealed that the key enzymes in folate synthesis include GTPCHI, ADCS, HPPK/DHPS, FPGS and GGS. GTPCHI, a homolog of the *folE* gene in *E. coli*, is the first enzyme of the de novo biosynthesis pathway of folates in bacteria, fungi, and plants. Hence, it has been mostly targeted for metabolic engineering because it is thought to be the first rate-determining step controlling flux into the folate pathway ^[47]. Similarly, ADCS catalyses the first step of pABA synthesis in plants.

Regarding the metabolic engineering of folates in legumes, a single study has been conducted in the common bean with no reported investigation in soybean. The Mexican common bean (*Phaseolus vulgaris* L.) was metabolically engineered by overexpressing GTPCHI, which enhanced folate levels in the seeds by 3-fold and pteridine levels by 150-fold ^[48]. In other studies, two key soybean folate biosynthesis genes, *GmGCHI* (GTPCHI) and *GmADCS* (ADCS) were cloned and co-overexpressed in maize and wheat ^[49]. Transgenic maize and wheat grains had folate content increased by 4.2-fold and 2.3-fold, respectively. A subsequent co-expression of GCHI from soybean and ADCS from tomato significantly increased folate levels in wheat (*Gm8gGCHI*⁺/*LeADCS*⁺).

Studies in other species and crops have shown that the metabolic engineering of folate biosynthesis genes enhances folate levels. The overexpression of the *folE* gene in microorganisms resulted in increased folate levels ^[50]. The ectopic expression of GTPCHI increased folate levels in rice by 3.3 to 6.1-fold ^[51], increased the folate content of tomato by an average of 2-fold and pteridine levels by 140-fold and increased lettuce folates from 2.1 to 8.5-fold ^[52]. A 1250-fold increase in pterins and a 2- to 4-fold enhancement of folates in *E. coli folE* overexpressed *Arabidopsis* plants have been reported ^[47]. Folate levels were also doubled by the overexpression of *E. coli folE* in a simultaneous biofortification study with vitamins β -carotenoid and vitamin C ^[53]. A 1.5–1.8-fold increase was reported in transgenic *AtADCS* lines ^[51].

Increasing the pteridine levels of tomato by the overexpression of GTPCHI resulted in the depletion of pABA, increased pteridine levels, and a 2-fold increase in folates ^[54]. The depleted pABA levels indicated that the pABA supply limits further accumulation. Further exogenous application of pABA in pABA-depleted crops increased the folate levels ^{[54][55]}. Thus, a combined engineering of p-ABA and pteridine production in tomatoes achieved 25-fold higher folates than controls and increased pABA and pteridine levels ^[56]. Similarly, the two-gene strategy for metabolic engineering of pterin and pABA in rice resulted in a 100-fold increase in folates ^[57]. However, co-overexpressing GTPCHI with ADCS could not increase folate content in potato and *Arabidopsis* ^[58], suggesting the need to engineer other pathways.

HPPK/DHPS is bifunctional and combines activities catalysing two consecutive steps to form dihydropteroate from HMDHP. HPPK/DHPS performs the condensation reaction of pterin and pABA in the mitochondria, while in *E. coli*, HPPK and DHPS are monofunctional enzymes, with their encoding genes being *folk* and *folP*, respectively. However, these enzymes are coupled as one protein in plants, protozoa, and fungi. Wheat HPPK/DHPS has been singly overexpressed in rice, which increased folate content 2-fold ^[59]. However, overexpressing *AtHPPK/DHPS* in rice resulted in no difference in folate levels ^[51].

FGPS is the enzyme that catalyses the attachment of the glutamate tail to the THF molecule. Polyglutamylation by FPGS is regarded as an essential regulatory point in folate metabolism $^{[60][61]}$. This is because the overexpression of GGH in animal systems reduces polyglutamate abundance and intracellular folate levels, whereas increased FPGS enhances intracellular folate levels. For instance, over-expression of GGH in *Arabidopsis* and tomato resulted in reduced folate levels $^{[61]}$. Thus, FGPS plays an important role in folate homeostasis. Overexpression of *AtFPGS* in rice increased seed folate content by 7.50 to 19.90% and 4.30–45.50% $^{[51]}$. In a recent study, the overexpression of foxtail millet FPGS gene *SiFPGS2* in *Arabidopsis* increased folate content $^{[62]}$. Owing to the previous unsuccessful attempt to enhance folates in potatoes by the two-gene approach (GTPCHI and ADCS), the four-gene approach overexpressing GTPCH1, ADCS, HPPK/DHPS and FPGS was studied, resulting in a 12-fold increase in folate content $^{[63]}$.

Soybean is a promising candidate for folate biofortification due to the variation and diversity of its folate content, as revealed in recent studies. Additionally, the availability of the soybean reference genome, along with advanced next-generation sequencing technologies and developed omic databases, has facilitated a deeper understanding of the genetics underlying various agronomic traits in soybean ^{[64][65]}. This understanding can be leveraged to develop new varieties with enhanced folate content through approaches such as conventional breeding, genomics-assisted breeding and metabolic engineering.

Some functional foods can be used for biofortification, such as fermented foods and sprouts. For example, traditional fermented soy-based foods, such as tempeh, natto, miso, soy sauce, douchi, and fermented soymilk, can help to increase the bioavailability of nutrients, making them more easily absorbed and used by the body [66][67][68]. Fermentation can also help to increase the overall nutrient content of these foods, as many microorganisms used in fermentation can synthesise essential vitamins and minerals. Fermentation has been shown to increase the folate content of some fermented soy-based foods such as tempeh and soymilk [10][11][69]. Similarly, soybean sprouts, which are made by germinating soybean seeds, are rich source of folates and contain higher amounts of the active component, 5MTHF ^[2]. By including these functional foods in the diet, it is possible to increase the intake of folate and other essential nutrients, helping to improve overall nutrition and address nutrient deficiencies.

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