RESEARCH PAPER

Improved folate accumulation in genetically modified maize and wheat

Qiuju Liang1,†, Ke Wang2,†, Xiaoning Liu1, Bisma Riaz2, Ling Jiang1, Xing Wan1, Xingguo Ye2,* and Chunyi Zhang1,∗

1 Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China
2 Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China
† These authors contributed equally to this work.
* Correspondence: zhangchunyi@caas.cn or yexingguo@caas.cn

Received 29 August 2018; Editorial decision 11 December 2018; Accepted 13 December 2018

Editor: Ariel Vicente, CONICET-National University of La Plata, Argentina

Abstract

Folates are indispensable co-factors for one-carbon metabolism in all organisms. In humans, suboptimal folate intake results in serious disorders. One promising strategy for improving human folate status is to enhance folate levels in food crops by metabolic engineering. In this study, we cloned two GmGCHI (GTP cyclohydrolase I) genes (Gm8gGCHI and Gm3gGCHI) and one GmADCS (aminodeoxychorismate synthase) gene from soybean, which are responsible for synthesizing the folate precursors pterin and p-aminobenzoate, respectively. We initially confirmed their functions in transgenic Arabidopsis plants and found that Gm8gGCHI increased pterin and folate production more than Gm3gGCHI did. We then co-expressed Gm8gGCHI and GmADCS driven by endosperm-specific promoters in maize and wheat, two major staple crops, to boost their folate metabolic flux. A 4.2-fold and 2.3-fold increase in folate levels were observed in transgenic maize and wheat grains, respectively. To optimize wheat folate enhancement, codon-optimized Gm8gGCHI and tomato LeADCS genes under the control of a wheat endosperm-specific glutenin promoter (1Dx5) were co-transformed. This yielded a 5.6-fold increase in folate in transgenic wheat grains (Gm8gGCHI+LeADCS+). This two-gene co-expression strategy therefore has the potential to greatly enhance folate levels in maize and wheat, thus improving their nutritional value.

Keywords: Aminodeoxychorismate synthase, biofortification, folates, GTP cyclohydrolase I, maize, wheat.

Introduction

Tetrahydrofolate and its derivatives are collectively termed folates and they belong to a group of water-soluble vitamin B compounds (B9) (Hanson and Gregory, 2002; Basset et al., 2005). As essential carriers and donors of one-carbon units, folates are involved in multiple metabolic processes, including biosynthesis of purine, thymidylate, methionine, serine, pantetheinate, and formylmethionyl-transfer RNA, providing methyl groups for most cellular methylation reactions (Scott et al., 2000). Folates play roles in histidine degradation pathways in mammals, including humans (Hilton et al., 2003). In addition, folates are necessary for biosynthesis of lignin, alkaloids, and chlorophyll (Hanson and Gregory, 2011). The significant roles played by folates indicate that they are indispensable micronutrients for all living organisms.

Microbes and plants can synthesize folates de novo, but mammals (including humans) lack the complete biosynthesis system and hence are unable to produce folates. Therefore, they must rely on dietary sources, mainly from plants. Folate levels vary among food sources. Eggs, liver, green leafy vegetables, and leguminous vegetables are high in folates, whereas some staple...
foods such as rice, wheat, and potatoes contain little (Bekaert et al., 2008; Blancquaert et al., 2010). Folate malnutrition is a global health problem that occurs even in developed countries. Folate deficiency increases the risk of many diseases, including infant neural tube defects, megaloblastic anemia, cardiovascular disease, and certain cancers (Li et al., 2003; Fenech, 2010; Nazki et al., 2014; Herrera-Araujo, 2016). Currently, fortified foods or synthetic folic acid pills are used to alleviate folate deficiency in Western countries (Blancquaert et al., 2014), but it would be difficult to implement such dietary intervention across all populations. Moreover, excessive uptake of synthetic folic acid has caused much concern. As our knowledge of folate biosynthesis and metabolism in plants has improved, engineering solutions for biofortification of food crops by enhancing their natural folate content have attracted much attention (Blancquaert et al., 2014; Strobbe and Van Der Straeten, 2017).

The folate biosynthesis pathways of Arabidopsis have been extensively investigated. At least 10 enzymes take part in this process, most of which have been cloned and functionally confirmed by in vitro assays or by complementation analysis (Ravanel et al., 2001; Basset et al., 2002, 2004; Sahr et al., 2006). Tetrahydrofolate (THF) is a tripartite molecule composed of pterin, p-aminobenzoate (pABA), and glutamate moieties. In plants, folate biosynthesis is highly compartmented; pterin and pABA are synthesized in the cytosol and plastids, respectively, and subsequent coupling of these two precursors and the addition of glutamate moieties takes place in mitochondria (Fig. 1) (Basset et al., 2005). GTP cyclohydrolase I (GCHI) and aminodeoxychorismate synthase (ADCS) act as the initial enzymes in the formation of pterin and pABA, respectively (Basset et al., 2002, 2004). It has been proposed that the reactions catalysed by these two enzymes act as rate-determining steps (Hossain et al., 2004).

The first attempt to increase folate content was by overexpression of GCHI. Overexpression of the folE gene encoding GCHI from E. coli in Arabidopsis yielded 1250-fold and ~2–4-fold increases in pterin and folate levels, respectively (Hossain et al., 2004). By inducing overexpression of the mammalian GCHI gene under fruit-specific promoters, pterin and folate contents of tomato fruit were increased by ~3–140 times and two times, respectively (Diaz de la Garza et al., 2004). This strategy was also applied to lettuce, corn, and common bean, resulting in 8.5–, 2–, and 3-fold increases in their folate contents, respectively (Naqvi et al., 2009; Nunes et al., 2009; Ramirez Rivera et al., 2016). Large increases in pterin levels of transgenic plants confirmed the key role of GCHI in promoting folate biosynthesis (Diaz de la Garza et al., 2004; Hossain et al., 2004). The relatively low levels of folate accumulation in these transgenic plants were presumed to be due to depletion of pABA, the other folate precursor, and further increases in folate levels were achieved by boosting pABA provision (Diaz de la Garza et al., 2004). A two-gene GCHI and ADCS simultaneous expression strategy proved very successful in tomato and rice, yielding 25-fold and 100-fold increases in folate levels, respectively (Diaz de la Garza et al., 2007; Storozhenko et al., 2007). Attempts to overexpress other folate biosynthesis genes in rice, or to couple them with GCHI, produced only slightly higher folate levels (Dong et al., 2014). A study of folate-biofortified rice indicated that its stability in storage can be improved by using animal folate-binding proteins (Blancquaert et al., 2015). A modest (3-fold) folate increase was observed in biofortified potatoes by co-expression of GCHI and ADCS (Blancquaert et al., 2014).
A greater folate enhancement (9-fold) was obtained by the additional introduction of the other two folate biosynthesis genes HPPK/DHPS (6-hydroxymethyl-2,4-pyridinephosphokinase/dihydrotrope synthase) and FPGS (folylpolyglutamate synthase) (De Lepeleire et al., 2018). These studies indicated the feasibility of folate biofortification in plant foods.

Soybeans (Glycine max) accumulate large amounts of folates. In contrast, the grains of maize and wheat accumulate them poorly. Therefore, we are seeking to use the folate biosynthesis genes from soybeans to improve folate contents of maize and wheat; however, the genes involved in soybean have not yet been functionally characterized. In this study, we isolated the two key soybean folate biosynthesis genes, GmGCHI and GmADCS, and confirmed their function in Arabidopsis. Previously, a 2-fold increase of folate content has been observed in white corn after overexpression of folE from E. coli (Naqvi et al., 2009). In this study we therefore enhanced the folate content of maize by achieving simultaneous expression of GmGCHI and GmADCS via endosperm-specific promoters. As folate biofortification in wheat has not been attempted previously, we co-expressed GCHI and ADCS from soybean and tomato to boost the folate metabolic flux. We observed significant enhancement of folate levels in the transgenic maize and wheat grains.

Materials and methods

Plant materials and growth conditions

The plant materials used in this study included the soybean (Glycine max) cultivar Zhonghuang24 provided by Prof. Wang Lei of the Biotechnology Research Institute of the Chinese Academy of Agricultural Sciences. We obtained the wheat (Triticum aestivum) cultivar Fielder from the National Crop Germplasm Bank at the Institute of Crop Science of the Chinese Academy of Agricultural Sciences. We obtained the Arabidopsis thaliana ecotype Columbia-0, the maize (Zea mays) cultivar line HiII and the maize inbred line Zheng58 from sources sustained in our laboratory.

The soybean plants were grown in a greenhouse at a temperature of 25 °C under 12/12 h light/dark conditions. We grew the Arabidopsis plants in an air-conditioned greenhouse maintained at a temperature of 22 °C. The light intensity was set to 300 μmol m–2 s–1 under a 16/8 h light/dark cycle. Immature wild-type embryos aged 10–12 d post anthesis were used for Agrobacterium-mediated transformation. The wheat plants were grown in a greenhouse at 22 °C under a 12/12 h light/dark cycle. Immature wild-type embryos at the flowering stage. The maize plants were grown in a sunlit greenhouse at 25 °C under a 12/12 h light/dark cycle. Immature wild-type embryos aged 10–12 d post anthesis were used for Agrobacterium-mediated transformation.

Expressed sequence tags used to obtain the target soybean genes

We BLASTed the Arabidopsis AtGCHI and AtADCS coding sequences against the soybean expressed sequence tag (EST) database on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and short and disconnected ESTs were found. There were four ESTs with high similarity to the AtGCHI 5' terminal sequence and 11 ESTs similar to the 3' terminal sequence. Two ESTs were similar to the 5' terminal sequence of AtADCS, one EST was similar to the AtADCS middle sequence, and eight ESTs were similar to the 3' terminal sequence (see Supplementary Fig. S1 at JXB online). We then used the resulting ESTs as a basis for assembling and amplifying the GmGCHI and GmADCS coding sequences. RNA extraction and gene full-sequence cloning by RACE

To clone the target genes, we collected young soybean leaves from 20-d-old seedlings, extracted total RNA using a UGENE Total RNA KIT II (Unipro) according to the manufacturer's instructions, and then used a First-Strand DNA Synthesis Kit (Toyobo) to synthesize single-stranded cDNAs. For cloning of GmGCHIs, first, two partial sequences (700 bp products, named M1, M2) were obtained using primers, namely GTPCHI-EST1-FW + GTPCHI-EST8-RV and GTPCHI-EST3-FW + GTPCHI-EST6-RV (Supplementary Figs S1B, S2A). Primer sequences are shown in Supplementary Table S1. Subsequently, we found that sequences of 5'EST-1, M1, and 3'EST-1/8 could overlap and be assembled into a 1200-bp sequence, and that 5'EST-3, M2, and 3'EST-6 could overlap and be assembled into a 1180-bp sequence. After a BLAST search in the genomic database of Glycine max, the two sequences were found to be respectively located in chromosome 8 (8g46160) and chromosome 3 (3g21540), and were named as Gm8gGCHI and Gm3gADCS. We used the 5' RACE and 3' RACE techniques to complete the GmGCHIs sequences. Total RNA was treated according to instructions provided by the manufacturer of the 3'-RACE System for Rapid Amplification of cDNA Ends and the 5'-RACE System for Rapid Amplification of cDNA Ends (Invitrogen). We designed the specific primers Gm8gGCHI5'RACE, Gm3gGCHI5'RACE nest, Gm8gGCHI3'RACE, Gm3gGCHI3'RACE nest, Gm3gGCHI3'RACE nest, and Gm3gGCHI8g3'RACE nest (Supplementary Fig. S1B, Table S1) and operated the PCR system following the manufacturer's instructions (Fig. S2B, C). We obtained a 66-bp 5' untranscribed region (UTR), a 1380-bp coding sequence (CDS), and 177-bp 3'-UTR for Gm3gGCHI, and a 323-bp 5'-UTR, a 1374-bp CDS, and 784-bp 3'-UTR for Gm8gGCHI. Finally, we obtained full coding sequences of the following GmGCHIs using primers: Gm3gGCHI1F1, Gm3gGCHI1R1, Gm8gGCHI1F1, and Gm8gGCHI1R1 (Supplementary Figs S1B, S2D). For amplification of GmADCS, a 1352-bp product was obtained with mESTF1+3'EST5-R2 (Supplementary Figs S1B, S2E). Following a BLAST search in the Glycine max database, the 1352-bp product was located in chromosome 10 (10g35580), and the location predicted a 2304-bp coding sequence. Based on this, a 2331-bp product was obtained with the primers ADCSMF + ADCSMR. Next, the full CDS sequence of GmADCS was obtained by RACE technology with the primers ADCS3'RACE, ADCS5'RACE nest, ADCS3'RACE, and ADCS5'RACE nest (Supplementary Figs S1B, S2E, G, Table S2) and it consisted of a 218-bp 5'-UTR, a 2784-bp CDS, and a 252-bp 3'-UTR. Finally, we obtained the full coding sequences of GmADCS with the primers ADCS10FLF1 and ADCS10R1 (Supplementary Table S2). We took the codon preferences of wheat as the basis for optimization of the full Gm8gGCHI and LeADCS coding regions, which were then synthesized directly by Jinsirui Biotech Co. (Nanjing).

Conserved domain analysis and phylogenetic tree of GmGCHI and GmADCS

We used the Conserved Domain Database (CDD) on the NCBI website (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to predict the conserved domains of the GmGCHI and GmADCS protein sequences. We used the ClustalW2 service (https://www.ebi.ac.uk/Tools/msa/clustalw2/) to perform multiple alignments of GCHI homologs and ADCS homologs. The phylogenetic trees were constructed using the MEGA4.0 software by use of the Neighbor-Joining analysis of Bootstrap Test of Phylogeney, with the Bootstrap set as 500 replicates.

Construction of GFP fusion proteins and subcellular localization analysis

We amplified full GmGCHI coding sequences with the terminating codon deleted and a 360-bp N terminal GmADCS sequence and cloned them in the correct direction into the pBgl II and Kpn I sites of a pRTL2 vector, respectively. The target sequences were fused with the
green fluorescent protein (GFP) coding regions. We isolated chloroplasts from Arabidopsis leaves grown in a greenhouse for 21 d and induced transient expression by applying the method described previously by Yoo et al. (2007). We captured the GFP signal using a laser-scanning confocal microscope (LSM700; Carl Zeiss).

**Construction and transformation of the overexpression vectors for Arabidopsis**

We cloned the full GmGCCHI and GmAADCSD coding sequences into the pCAMBIA1301 binary vector construct at the Nco I and Pml I sites, respectively, under the control of the CaMV35S promoter. These constructs were transformed into the Arabidopsis wild-type by the Agrobacterium tumefaciens method (Zhang et al., 2006). T1 plants were harvested and plated on half-strength Murashige–Skoog culture medium supplied with 25 mg l−1 hygromycin B, and the surviving green seedlings were transplanted on half-strength Murashige–Skoog culture medium supplied from Arabidopsis leaves grown in a greenhouse for 21 d and induced green fluorescent protein (GFP) coding regions. We isolated chloroplasts from the transgenic plants. Total RNA was extracted with an RNA Extraction Kit supplied by Yuanchao Biotech Co. (Tianjin, China) and cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Fermentas). The cDNA templates were subjected to PCR using the primers Gm8gGCCHI-FSP and Gm8gGCCHI-RSP, and GmAADCSD-FSP and GmAADCSD-RSP (Supplementary Tables S1, S2) under the following conditions: 94 °C for 5 min, then 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s for 32 cycles. We used wheat and maize Actin genes as internal controls and performed PCR amplification using the following primers: TmActinF1, TmActinR1, ZmActinF1, and ZmActinR1 (Supplementary Table S2) and the same PCR conditions as above, but with only 28 cycles. The PCR products were analysed in a 1% agarose gel and photographed using a Peiqing gel system (Shanghai, China). We applied a haploid-doubling technique via anther culture (Yin et al., 2018) to obtain homozygous transgenic wheat plants, then confirmed successful completion by fluorescence in situ hybridization according to methods described previously (Guo et al., 2016).

We confirmed successful integration of the optimized Gm8gGCCHI and LeAADCSD sequences into transgenic wheat plants by PCR amplification of their complete coding regions using the primers Gm8gGCCHI-FSP, Gm8gGCCHI-RSP, LeAADCSD-FSP, and LeAADCSD-RSP (see Supplementary Fig. S1B, Tables S1, S2). Homozygous T2 transgenic plants were used for folate detection analysis. We obtained Arabidopsis plants containing both the GmGCCHI and the GmAADCSD genes by crossing the GmGCCHI-overexpressing plants with the GmAADCSD-overexpressing plants.

**Construction and transformation of overexpression vectors for maize and wheat**

To support stable transformation of maize and wheat, we used a pCAMBIA1301 vector containing the phosphinotrin-resistant (PPT) resistance gene as a basic vector system. Digestion with the two blunt-end enzymes Spal I and Pml I and self-ligation resulted in deletion of the 355 promoter and GUS sequences from the pCAMBIA1301 vector. The full GmAADCSD coding sequence was introduced into a pH20754 vector that contained the maize endosperm-specific promoter LgαIA and the LgαIA terminal sequences, at the Nco I and Pml I digestion sites. The full CDS sequences of the Gm8gGCCHI gene were cloned into the pH20754 vector at the Nco I and Pml I sites, and then 2109 bp of the rice endosperm-specific promoter GluC was cloned into the vector at the EcoRV and Nco I sites to substitute the primate maize LgαIA promoter. Next, the Gm8gGCCHI expression cassette was cloned into the GmAADCSD-pH20754 vector at the Smal I site. Finally, the complete Gm8gGCCHI and GmAADCSD cassettes were introduced into the truncated pCAMBIA1301 vector at the BstE II site.

The complete Gm8gGCCHI-GmAADCSD-pCAMBIA1301 vector was transformed into A. tumefaciens strain EHA105 and introduced into maize cultivar Hil1 as described previously (Frame et al., 2002). The same vector was transformed into A. tumefaciens C58C1 and introduced into the wheat cultivar Fielder genome by methods described previously (Ishida et al., 2015; Wang et al., 2017).

Codon-optimized Gm8gGCCHI and LeAADCSD genes were cloned onto a double T-DNAs vector pWM122 driven by a wheat endosperm-specific glutenin (1DS)5 promoter (Wang et al., 2017) and transformed into A. tumefaciens C58C1 and introduced into immature embryos of the wheat cultivar Fielder using methods described previously (Ishida et al., 2015; Wang et al., 2017).

**Identification of transgenic maize and wheat plants**

Positive maize and wheat transient seedlings were analysed by PCR using the method described above for detecting transgenic Arabidopsis plants. We achieved stable inheritance by transferring the T-DNA expression cassette into a background genetic material of the maize inbred line Zheng58 by pollinating the transgenic maize Hil1 cultivar with Zheng58 pollen. We produced generations BC1~BC5 after backcrossing with Zheng58 five times. We confirmed successful production of stable transgenic maize lines by applying a previously described Southern blotting method (Wang et al., 2017), then conducted RT-PCR analysis to detect the transscripts of the target genes in immature 25-d-old kernels of the transgenic plants. We captured the signal of the transscripts of the target genes in immature 25-d-old kernels of the transgenic plants. Total RNA was extracted with an RNA Extraction Kit supplied by Yuanchao Biotech Co. (Tianjin, China) and cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Fermentas). The cDNA templates were subjected to PCR using the primers Gm8gGCCHI-FSP and Gm8gGCCHI-RSP, and GmAADCSD-FSP and GmAADCSD-RSP (Supplementary Tables S1, S2) under the following conditions: 94 °C for 5 min, then 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s for 32 cycles. We used wheat and maize Actin genes as internal controls and performed PCR amplification using the following primers: TmActinF1, TmActinR1, ZmActinF1, and ZmActinR1 (Supplementary Table S2) and the same PCR conditions as above, but with only 28 cycles. The PCR products were analysed in a 1% agarose gel and photographed using a Peiqing gel system (Shanghai, China). We applied a haploid-doubling technique via anther culture (Yin et al., 2018) to obtain homozygous transgenic wheat plants, then confirmed successful completion by fluorescence in situ hybridization according to methods described previously (Guo et al., 2016).

We confirmed successful integration of the optimized Gm8gGCCHI and LeAADCSD sequences into transgenic wheat plants by PCR amplification of their complete coding regions using the primers Gm8gGCCHI-FSP, Gm8gGCCHI-RSP, LeAADCSD-FSP, and LeAADCSD-RSP. We obtained the transscripts of target genes in the pollinated, immature, 25-d-old wheat kernels by RT-PCR using the primers Gm8gGCCHI-FSP, Gm8gGCCHI-RSP, LeAADCSD-FSP, LeAADCSD-RSP, and TmActinF1, TmActinR (Supplementary Tables S1, S2) under the same PCR conditions as described above.

**Determination of levels of folate and its precursors contained in transgenic plants**

We collected Arabidopsis leaves after 30 d growth in a greenhouse and mature maize and wheat grains after desiccating them for 48 h at 37 °C, and measured their folate, pterin, and pABA contents using a previously described method with some modifications (Lu et al., 2007). Briefly, we used ~0.05 g of the tissues and ground them to a powder in liquid nitrogen. We immediately added 1000 μl of freshly prepared extraction buffer (5 mM phosphate buffer containing 0.5% sodium ascorbate and 0.1% 2-mercaptoethanol, pH 7.2) to the powder via a 1.5-ml tube before thawing the sample. After homogenizing the mixture, we immediately boiled it for 10 min, cooled it on ice for 10 min, and centrifuged it at 16 000 g at 4 °C for 10 min. We deconjugated the polyglutamylated tails by adding 35 μl of rat serum to the supernatant and incubating it at 37 °C for 4 h. The samples were then boiled again for 10 min, cooled on ice for 10 min, and centrifuged at 16 000 g at 4 °C for 10 min. The supernatant was subjected to 3-kDa ultra-filtration and then used for LC/MS/ MS analysis.

Chromatographic analyses were performed on a 1260 HPLC system (Agilent) using an Akzo Nobel analytical column (Kromasil 100–5 C18, 50×2.1 mm) at a flow rate of 0.30 ml min−1. The injection volume was 15.0 μl. The temperature of the injector and column oven were separately maintained at 4 °C and 25 °C, respectively. The mobile phases were 0.1% (v/v) formic acid in water (phase A) and 0.1% (v/v) formic acid in acetonitrile (phase B). The gradient program was a total of 19 min. The proportion of mobile phase B was increased linearly from 5 to 9% over 2 min. In the following 6 min, phase B increased to 9.6% and was then sharply increased to 20% over 0.2 min. After holding at 20% for 3 min, the proportion of phase B was decreased to 5% in 0.2 min followed by a subsequent equilibration. An Agilent 6420 triple-quadrupole tandem MS coupled to an ESI (electron spray ionization) interface was used for mass analyses and quantification of target analytes. The mass spectrometer was operated in positive ion mode. The parameters were optimised for the target analytes with a gas temperature of 350 °C, drying gas flow at 11 l min−1, nebuliser pressure at 35 psi, and capillary voltage at 3500 V (+). System operation, data acquisition and data analyses were performed using the MassHunter software. Hydroxy methylpterin (m/z 194–106, 30 eV), pterin-6-carboxylic acid (m/z 208–162, 20 eV), p-aminobenzoate (pABA, m/z 138–65, 30 eV), 5,10-methenyltetrahydrofолate (5,10-CH2=THF, m/z 456–412, 30 eV), 5-methyltetrahydrofolate (5-M-THF, m/z 460–313, 20 eV), 5-formyltetrahydrofolate (5-F-THF, m/z 474–327, 20 eV), tetrahydrofolate (THF, m/z 446–299, 20 eV), and
methotrexate (MTX, m/z 455–308, 30 eV) purchased from Schirck Laboratories (Jona, Switzerland) were used as standards. We repeated each sample preparation five times.

Statistical analysis
Five biological replicates were used for detection analysis of each sample and results are shown as the means of all biological replicates. Statistical analysis of data was performed using t-tests in Microsoft Excel 2010.

Accession numbers
Tomato GCHI (NP_001234141), Arabidopsis thaliana GCHI (AT3G07270), Homo sapiens GCHI (NP_000152), E. coli folE (NP_288736), tomato ADCS (NP_001234467), Arabidopsis thaliana ADCS (AT2G28880), and E. coli pabB (AGEC1).

Results
Cloning of the GmGCHI and GmADCS genes from soybean
Leguminous plants such as soybean are good sources of folate. Therefore, it is important to be able to clone the coding regions of GCHI and ADCS from soybean for genetic modification of cereal plants. By using the Arabidopsis AtGCHI and AtADCS coding sequences as query sequences, we found several short and disconnected ESTs related to soybean GCHI and ADCS (Supplementary Fig. S1). Partial coding sequences of two GmGCHI genes and one GmADCS gene were assembled and amplified using specifically designed primers (Supplementary Tables S1, S2, Fig. S2). We then applied a RACE technique to obtain the full coding sequences of GmGCHI and GmADCS.

The two GmGCHI genes were located on chromosome 3 (3g21540) and chromosome 8 (8g46160), and were named Gm3gGCHI and Gm8gGCHI, respectively, and GmADCS was located on chromosome 10 (10g35580).

We characterized the GmGCHI and GmADCS sequences by analysing their predicted proteins. GCHIs have been cloned in E. coli, yeast, and mammalian samples, and subsequent crystal structure analysis has shown that E. coli and human GCHIs are homodecamers with ~26-kDa subunits, comprised of five tightly associated dimers (Nar et al., 1995; Nardese et al., 1996; Auerbach et al., 2000). In plants, tomato and Arabidopsis GCHIs have been identified as ~50-kDa proteins that contain two GCHI-like domains in tandem. Neither of the domains interacts with folate precursors and folate in Arabidopsis leaves. As shown in Fig. 4, we observed the GFP signals of the two GmGCHI fusion proteins in cytosol and the GFP signal of the GmADCS-N fusion protein in chloroplasts. The subcellular localization of GmGCHI and GmADCS was consistent with the compartmentation of folate biosynthesis, in which GCHI is involved in pterin biosynthesis in the cytosol and ADCS is involved in pABA formation in plastids.

Subcellular localization of GmGCHI-GFP and GmADCS-N-GFP fusion proteins
Given that the folate biosynthesis pathways of plants are compartmented, we analysed the subcellular localization of the three proteins described above by protoplast transient transformation. For this purpose, we fused the full coding sequences of two GmGCHI genes and the N-terminal sequence (360 bp) containing the predicted signal peptide sequence of the GmADCS gene with GFP protein, respectively, under the control of the CaMV35S promoter. The three constructs and the control vector were introduced into protoplasts isolated from Arabidopsis leaves. As shown in Fig. 4, we observed the GFP signals of the two GmGCHI fusion proteins in cytosol and the GFP signal of the GmADCS–N fusion protein in chloroplasts. The subcellular localization of GmGCHI and GmADCS was consistent with the compartmentation of folate biosynthesis, in which GCHI is involved in pterin biosynthesis in the cytosol and ADCS is involved in pABA formation in plastids.

Effects of GmGCHI and GmADCS overexpression on levels of folate precursors and folate in Arabidopsis
To assess the specific roles that the isolated GmGCHI and GmADCS genes play in folate biosynthesis, we introduced the entire coding regions of GmGCHIs and GmADCS driven by CaMV35S promoters into wild-type Arabidopsis (Fig. 5A). We obtained transgenic plants overexpressing Gm3gGCHI (3gGCHI-OE), Gm8gGCHI (8gGCHI-OE), and GmADCS (ADCS-OE). Pterin contents of GCHI-OE plants were significantly greater than those of the wild-type (Fig. 5B). An average 22-fold increase in pterin content (mainly of hydroxy- methylenopterin and pterin-6-carboxylic acid) was observed in 3gGCHI-OE plants and ~79–102-fold in 8gGCHI-OE plants (P<0.01). The average free pABA content of ADCS-OE plants overexpressing GmADCS was 2-fold greater than that of the wild type (P<0.01). However, folate levels of GCHI-OE plants did not differ significantly from those of the wild-type and were slightly lower in ADCS-OE plants (Fig. 5B). We then crossed the two types of transgenic Arabidopsis plants, thereby obtaining GA (GCHI-OEs/ADCS-OEs) plants, to evaluate the effect of the two-gene co-overexpression on folate accumulation. Folate levels of 3gGCHI-OE/GmADCS-OE cross-plants were ~1.2–1.3 times higher than those of the wild-type. Furthermore, we obtained greater increases in folate (~1.4–1.9 times) in the crosses with Gm8gGCHI overexpression (P<0.05) (Fig. 5C, D). The results therefore suggest that co-expression of GmGCHI and GmADCS provides a more
Fig. 2. Schematic characterization of GmGCHIs and multiple alignments with other homologs. (A) Conserved domains of the deduced GmGCHI proteins. There are two tunneling fold (T-fold) superfamilies in GmGCHIs, localized at ~35–185 aa and ~267–453 aa in Gm3gGCHI, and at ~24–176 aa and ~265–448 aa in Gm8gGCHI. (B) Alignment of GCHI homologs of soybean (Gm), tomato (Le), Arabidopsis thaliana (At), Homo sapiens (Hs), and E. coli (Ec). Identical and similar residues are shaded in black and gray, respectively. Dashes are gaps that maximize the alignment. (C) Phylogenetic analysis of GCHI homologs by use of Neighbor-Joining analysis.
Fig. 3. Schematic characterization of GmADCS and multiple alignments with other homologs. (A) Conserved domains of the deduced GmADCS. The GmADCS protein contains three domains including the GAT_1 superfamily (type 1 glutamine amidotransferase), Anth_synt_I_N (anthranilate synthase component I, N terminal region), and Chorismate_bind superfamily. (B) Alignment of ADCS homologs of soybean (Gm), tomato (Le), Arabidopsis thaliana (At), and E. coli (EcpabB). Identical and similar residues are shaded in black and gray, respectively. Dashes are gaps that maximize the alignment. (C) Phylogenetic analysis of ADCS homologs by use of Neighbor-Joining analysis.
Fig. 4. Subcellular localization of GmGCHI-GFP and GmADCS-N-GFP fusion proteins in Arabidopsis mesophyll protoplasts. The coding regions of two GmGCHI genes and a 300-bp N-terminal sequence of GmADCS were fused with GFP at the C-terminal. The cytoplasm localization of GFP was used as the control; the GFP signal is indicated in green and the chlorophyll autofluorescence (Chl) in red. As the images show, the two GmGCHI fusion proteins were localized to the cytosol with similar signals to GFP. The GmADCS-N terminal fusion protein localized in the chloroplasts as the green signal merges completely with the Chl. The images were scanned using a confocal microscope. The scale bars represent 5 μm.

Fig. 5. Analysis of folate, pterin, and pABA in transgenic Arabidopsis leaves. (A) Schematic representation of the T-DNA regions of the expression vectors used in Arabidopsis transformation. The coding regions of GmGCHI and GmADCS were driven by CaMV35S promoters (35S). (B) Pterin, pABA, and folate levels in Arabidopsis wild-type (WT), Gm3gGCHI overexpression plants (3gGCHI-OEs), Gm8gGCHI overexpression plants (8gGCHI-OEs), and GmADCS overexpression plants (ADCS-OEs). (C) Folate analysis in crossed plants (GAs) of GCHI-OEs/ADCS-OEs. (D) The crossing combinations of GA plants. Significant differences compared with the wild-type were determined using Student’s t-test: *P<0.05; **P<0.01.
Co-expression of GmGCHI and GmADCS in maize and wheat

We investigated whether it is potentially feasible to implement folate biofortification by simultaneously engineering the pterin and pABA branches in maize and wheat. The sequences encoding the GmGCHI and GmADCS genes were driven by endosperm-specific promoters from the rice GluC gene and maize LegIA gene, respectively, and were linked to the expression vector pCAMBIA3301 in a single T-DNA region (Fig. 6A). We applied an Agrobacterium-mediated transformation to introduce the GmGCHI-GmADCS-pCAMBIA3301 vector into the maize cultivar HiII. In addition, the T-DNA expression cassette was introduced into the maize inbred line Zheng58 by backcrossing, until the BC5 generation was obtained. We confirmed that the exogenous folate genes were integrated into the genome as a single copy by Southern blotting (Fig. 6B) and the introduced genes were abundantly expressed by RT-PCR (Fig. 6C). We evaluated the folate concentrations of mature grains in transgenic maize, and used grains from non-transgenic plants segregated from the heterozygous transgenic plants as controls. The average folate content of transgenic plants was ~3–4.2 times greater than that of the control plants (Fig. 6D). For example, the folate levels were significantly enhanced in transgenic maize 19–26 in comparison with the control (3.43±0.25 versus 0.82±0.09 nmol g⁻¹ dry weight; P<0.01). Meanwhile, we detected very high pterin contents, mainly of hydroxymethylpterin and pterin-6-carboxylic acid, in transgenic maize. The pterin contents were up to ~10–17 times higher than those of the control (P<0.01). Unexpectedly, there were no large differences in the pABA content between the control and transgenic maize (Fig. 6D). 5-Methyltetrahydrofolate (5-M-THF) and 5-formyltetrahydrofolate (5-F-THF) are the two major folate derivatives found in maize grains. The 5-M-THF and 5-F-THF levels were significantly higher in transgenic grains than in the control grains (Fig. 6E). Specifically, 5-M-THF was elevated most, accounting for up to 82% of the total folate content in transgenic maize 19–26.

We then introduced the Gm8gGCHI-GmADCS-pCAMBIA3301 co-expression vector into the wheat cultivar Fielder by Agrobacterium-mediated transformation and homozygous transgenic plants were obtained using the
haploid-doubling technique. We confirmed the insertion of the T-DNA cassette by fluorescence in situ hybridization in homozygous plants (Fig. 7A). RT-PCR detected high levels of Gm8gGCHI and GmADCS expression in the developing grains of the transgenic wheat (Fig. 7B). Folate analysis of the mature grains revealed that the average folate contents of homozygous transgenic wheat were 2.3 times higher than those of the wild-type (1.49±0.11 versus 0.63±0.05 nmol g⁻¹ dry weight, ~65 μg/100 g versus ~28 μg/100 g; P<0.01) (Fig. 7C). Moreover, we detected a ~2.6–4.7-fold increase in the free pABA levels and a ~1.8–4.0-fold increase in the pterin levels (mainly of hydroxymethylpterin and pterin-6-carboxylic acid) in transgenic grains (P<0.01) (Fig. 7C). 5-M-THF and 5-F-THF were the major folate derivatives in wheat grains. For example, 5-M-THF and 5-F-THF constituted 46% and 16%, respectively, of the total folate in transgenic wheat K28 (Fig. 7D).

**Co-expression of codon-optimized GmGCHI and tomato ADCS in wheat**

We investigated the possibility of further enhancing the folate content of wheat grains by use of the codon-optimized tomato gene LeADCS and soybean gene Gm8gGCHI. The two target genes, controlled by a wheat endosperm-specific glutenin (1Dx5) promoter, were constructed into the vector pWMB122, which contained double T-DNAs to obtain marker-free transgenic plants (Fig. 8A). We then co-introduced the two vectors Gm8gGCHI-pWMB122 and LeADCS-pWMB122 to the wheat cultivar Fielder by Agrobacterium-mediated transformation. PCR detection of the target genes identified three types of transgenic plants carrying either Gm8gGCHI⁺ (G) or LeADCS⁺ (A), or both Gm8gGCHI⁺ and LeADCS⁺ (GA) (Fig. 8B), and RT-PCR analysis revealed high expression of Gm8gGCHI and LeADCS in the transgenic wheat grains (Fig. 8C).

We assessed the levels of folates and their precursors in the mature grains of the transgenic wheat plants, again using the non-transgenic plants segregated from heterozygous transgenic plants as controls. The levels of the folate precursor pterin were up to 16 times higher in the G grains than that of the control (P<0.01); however, no significant changes were observed in pABA levels and folates were increased only slightly (1.4-fold). pABA was increased by up to 12-fold in the A grains (P<0.01); however, no significant changes in pterin levels were detected, and only a slight increase (1.3-fold) in folate content was observed, compared to the control. In GA plants containing both the target genes (Gm8gGCHI⁺/LeADCS⁺), folate levels...
Improved folate accumulation in genetically modified maize and wheat | 1549

Fig. 8. Folate engineering in wheat by optimized GmGCHI and LeADCS. (A) Schematic representation of the T-DNA regions of the vector used in wheat transformation. (B) Identification of three types of transgenic wheat by PCR amplification of the target genes using genomic DNA as templates: GmbGCHI+ (G), LeADCS+ (A), and GmbGCHI+/LeADCS+ (GA). Non-transgenic wheat is indicated as ‘negative’. (C) Expression levels of GmbGCHI and GmADCS in transgenic wheat kernels. (D) Folate, pterin, and pABA levels and (E) the main folate derivatives in transgenic wheat grains and grains of the negative wheat without the target genes. Significant differences compared with the ‘negative’ control were determined using Student’s t-test: *P<0.05; **P<0.01.

increased by up to 5.6-fold (2.91±0.19 versus 0.52±0.05 nmol g⁻¹ dry weight for GA and controls grains, respectively; P<0.01) (Fig. 8D). In addition, the concentrations of pterin (mainly hydroxymethylpterin and pterin-6-carboxylic acid) and free pABA in the GA grains increased by ~4.7–10-fold and ~2.4–8 fold, respectively, compared to those of the control. 5-M-THF and 5-F-THF; the two major folate species, accounted for 67% and 20% of the total folates in the transgenic grains, respectively, and were significantly increased in the transgenic wheat GA grains (Fig. 8E).

Discussion

Enhancing folate accumulation in food crops by applying metabolic engineering is regarded as an important strategy with great promise for alleviating folate malnutrition (Blancquaert et al., 2014). GTP cyclohydrolase I (GCHI) and aminodeoxychorismate synthase (ADCS) are two key enzymes that catalyse the formation of the folate precursors pterin and p-aminobenzoate, respectively, and the reactions that they perform are also considered as rate-limiting steps in folate biosynthesis (Díaz de la Garza et al., 2007; Storozhenko et al., 2007). In this study, biofortified maize and wheat with improved folate levels were created by co-overexpression of these two genes. First, we successfully cloned the soybean GmGCHI and GmADCS genes by combining EST-based amplification and the RACE technique (Supplementary Figs S1, S2). We then conducted a transgenic analysis of the two soybean genes in Arabidopsis, which confirmed that their roles in the biosynthesis of the folate precursors pterin and pABA were conserved. This provides the first report of the cloning and characterization of genes involved in soybean folate biosynthesis. The genes were then used in metabolic engineering.

Folate enhancement in transgenic Arabidopsis plants was not observed with the single genes GmGCHI+ or GmADCS+; however, a ~1.2–1.9-fold increase was achieved when both genes were carried (Fig. 5). Similarly, the folate levels of transgenic wheat grains expressing the single genes GmGCHI+ or LeADCS+ were only increased by 1.4-fold, whereas levels in GmGCHI+/LeADCS+ wheat grains were increased by 5.6-fold (Fig. 8). We therefore conclude that the two-gene strategy of simultaneously boosting both pterin and pABA has greater potential for enhancing folate accumulation in Arabidopsis and wheat than a single-gene strategy.
Folate biofortification via overexpression of the *GCHI* and *ADCS* genes has been very successful in tomato (25-fold) and rice (100-fold) (Díaz de la Garza et al., 2007; Storozenko et al., 2007), and has had modest success in potato (3-fold) (Blancquaert et al., 2013). In our study, we increased the folate contents of maize and wheat by ~2–6-fold. Taking all these findings together, we presume that regulation of folate biosynthesis varies among plant species. Other possible limiting factors are predicted to affect the potential of folate biofortification in plants. In *GCHI*/*ADCS* potatoes, introduction of the genes HPPK/DHPS and FPGS augmented folate levels up to 9-fold (De Lepeleire et al., 2018); thus, the bottle-necks were predicted to arise from other folate biosynthesis genes. Similar to *GCHI*/*ADCS* potatoes (Blancquaert et al., 2013), we also observed considerable increases in the folate precursors pterin and pABA in transgenic wheat grains with *GmGCHI*/*LeADCS* (Fig. 8C) but only modest increases in folate levels. Therefore, there may be other limiting factors that affect folate biosynthesis in wheat. In the case of maize, the transgene expression increased folates and pterin by ~3–4.2 fold and ~10–17 fold, respectively, but it had no impact on pABA levels (Fig. 6D). This observation led us to conclude that the pABA branch might be more tightly regulated than the pterin branch in maize, thus resulting in the modest enhancement of folates. In contrast, an increase in the pterin branch has been shown to cause a significant rise in pABA levels in genetically engineered common bean (Ramírez Rivera et al., 2016). Thus, it seems that endogenous regulation of folate biosynthesis in plants is somewhat complicated and a case-by-case approach should be taken when folate biofortification is pursued via metabolic engineering.

The biosafety of genetically modified crops remains an issue of great public concern. It would be helpful to remove selectable markers from transgenic plants, as this would alleviate public concerns regarding genetically engineered crop varieties escaping into local ecosystems (Wang et al., 2012; Tuteja et al., 2012; Wang et al., 2017), and double T-DNA-mediated co-transformation has been successfully applied for this purpose in many species (Xing et al., 2000; Miller et al., 2002; Lu et al., 2009; Ramana Rao et al., 2011; Wang et al., 2017). In our study, transgenic wheat plants with significant improvement of folate accumulation were generated by co-transformation of folate genes with the selectable marker gene *bar* (Fig. 8). In future studies, we will focus on transgenic events that do not carry *bar* gene.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Blast of soybean ESTs with the *ArGCHI* and *AtADCS* coding sequences and primer maps for cloning and identification of *GmGCHI* and *GmAADC*. 

Fig. S2. Amplification of *GmGCHI* and *GmAADC*.

Table S1. Primers used in cloning and identification of *GmGCHI* genes.

Table S2. Primers used in cloning and identification of *GmAADC* and *LeADCS*.

**Acknowledgements**

This work was supported by the National Special Program for Transgenic Research (2016ZX08003-002), the Ministry of Science and Technology of China (2016YFD0100503), the Shanghai Agriculture Applied Technology Development Program (Z20180103), and the Collaborative Innovation Action, Agricultural Science and Technology Innovation Program of Chinese Academy of Agricultural Sciences (CAAS-XTCX2016009). The authors declare that they have no conflicts of interest.

**Author contributions**

QL and KW designed the study; XY and CZ supervised the research; QL and XL performed the cloning of the target genes, obtained the transgenic Arabidopsis plants, and conducted the expression pattern analysis; QL performed the maize transformation, and identified and cultivated the transgenic maize plants; KW, RB, and XY performed the wheat transformation, and identified and cultivated the transgenic wheat plants; QL, LJ, and XW carried out the folate extraction and detection; QL, KW, XY, and CZ performed the data analysis and prepared the manuscript; all of the authors read and agreed on the final manuscript.

**References**

Auerbach G, Herrmann A, Bracher A, et al. 2000. Zinc plays a key role in human and bacterial GTP cyclohydrolase I. Proceedings of the National Academy of Sciences, USA 97, 13567–13572.

Basset GJ, Quinlivan EP, Gregory JF 3rd, Hanson AD. 2005. Folate synthesis and metabolism in plants and prospects for biofortification. Crop Science 45, 449–453.

Basset GJ, Quinlivan EP, Ravanel S, et al. 2004. Folate synthesis in plants: the p-aminobenzoate branch is initiated by a bifunctional PabA-PabB protein that is targeted to plastids. Proceedings of the National Academy of Sciences, USA 101, 1496–501.

Basset GJ, Quinlivan EP, Ziemak MJ, Díaz De La Garza R, Fischer M, Schiffmann S, Bacher A, Gregory JF 3rd, Hanson AD. 2002. Folate synthesis in plants: the first step of the pterin branch is mediated by a unique bimodular GTP cyclohydrolase I. Proceedings of the National Academy of Sciences, USA 99, 12489–12494.

Bekaert S, Storozenko S, Mehrshahi P, Bennett MJ, Lambert W, Gregory JF 3rd, Schubert K, Hugenholz J, Van Der Straeten D, Hanson AD. 2008. Folate biofortification in food plants. Trends in Plant Science 13, 28–35.

Blancquaert D, De Steur H, Gellynck X, Van Der Straeten D. 2014. Present and future of folate biofortification of crop plants. Journal of Experimental Botany 65, 895–906.

Blancquaert D, Storozenko S, Loizeau K, De Steur H, De Brouwer V, Vlaene J, Ravanel S, Rebeille F, Lambert W, Van Der Straeten D. 2010. Folates and folic acid: from fundamental research toward sustainable health. Critical Reviews in Plant Sciences 29, 14–35.

Blancquaert D, Storozenko S, Van Daele J, Stove C, Visser RG, Lambert W, Van Der Straeten D. 2013. Enhancing pterin and para-aminobenzoate content is not sufficient to successfully biofortify potato tubers and Arabidopsis thaliana plants with folate. Journal of Experimental Botany 64, 3899–3909.

Blancquaert D, Van Daele J, Strobbe S, Kieksen F, Storozenko S, De Steur H, Gellynck X, Lambert W, Stove C, Van Der Straeten D. 2015. Improving folate (vitamin B9) stability in biofortified rice through metabolic engineering. Nature Biotechnology 33, 1076–1078.

De Lepeleire J, Strobbe S, Verstraete J, Blancquaert D, Ambach L, Visser RGF, Stove C, Van Der Straeten D. 2018. Folate biofortification
of potato by tuber-specific expression of four folate biosynthesis genes. Molecular Plant 11, 175–188.

Díaz de la Garza RI, Gregory JF 3rd, Hanson AD. 2007. Folate biofortification of tomato fruit. Proceedings of the National Academy of Sciences, USA 104, 4218–4222.

Díaz de la Garza R, Quinlivan EP, Klaus SM, Basset GJ, Gregory JF 3rd, Hanson AD. 2004. Folate biofortification in tomatoes by engineering the pteridine branch of folate synthesis. Proceedings of the National Academy of Sciences, USA 101, 13720–13725.

Dong W, Cheng ZJ, Lei CL, et al. 2014. Overexpression of folate biosynthesis genes in rice (Oryza sativa L.) and evaluation of their impact on seed folate content. Plant Foods for Human Nutrition 379–385.

Díaz de la Garza RI, Gregory JF 3rd, Hanson AD. 2004. Folate biofortification of tomato fruit. Proceedings of the National Academy of Sciences, USA 101, 13720–13725.

Fenech M. 2010. Folate, DNA damage and the aging brain. Mechanisms of Ageing and Development 131, 236–241.

Frame BR, Shou H, Chikwamba RK, et al. 2002. Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system. Plant Physiology 129, 13–22.

Guo X, Su H, Shi Q, Fu S, Wang J, Zhang X, Hu Z, Han F. 2016. De novo centromere formation and centromeric sequence expansion in wheat and its wide hybrids. Plos Genetics 12, e1005997.

Hanson AD, Gregory JF 3rd. 2002. Synthesis and turnover of folates in plants. Current Opinion in Plant Biology 5, 244–249.

Hanson AD, Gregory JF 3rd. 2011. Folate biosynthesis, turnover, and transport in plants. Annual Review of Plant Biology 62, 105–125.

Herrera-Araujo D. 2016. Folic acid advisories: a public health challenge? Health Economics 25, 1104–1122.

Hilton JF, Christensen KE, Watkins D, Raby BA, Renaud Y, de la Luna S, Estivill X, MacKenzie RE, Hudson TJ, Rosenblatt DS. 2003. The molecular basis of glutamate formiminotransferase deficiency. Human Mutation 22, 67–73.

Hossain T, Rosenberg I, Selhub J, Kishore G, Beachy R, Schubert K. 2004. Enhancement of folates in plants through metabolic engineering. Proceedings of the National Academy of Sciences, USA 101, 5158–5163.

Ishida Y, Tsunashima M, Hiei Y, Komari T. 2015. Wheat (Triticum aestivum L.) transformation using immature embryos. Methods in Molecular Biology 1223, 189–198.

Li GM, Presnell SR, Gu L. 2003. Folate deficiency, mismatch repair-dependent apoptosis, and human disease. The Journal of Nutritional Science and Vitaminology 49, 1–8.

Li J, Ye X, An B, Du L, Xu H. 2012. Genetic transformation of wheat: current status and future prospects. Plant Biotechnology Reports 6, 183–193.

Lu L, Wu XR, Yin XY, Morrand J, Chen XL, Folk WR, Zhang ZJ. 2009. Development of marker-free transgenic sorghum [Sorghum bicolor (L.) Moench] using standard binary vectors with bar as a selectable marker. Plant Cell Tissue and Organ Culture 97, 98–108.

Lu W, Kwon YK, Rabinowitz JD. 2020. Improved folate accumulation in genetically modified maize and wheat | 1551

Nar H, Huber R, Auerbach G, Fischer M, Hösl C, Ritz H, Bracher A, Meining W, Eberhardt S, Bacher A. 1995. Active site topology and reaction mechanism of GTP cyclohydrolase I. Proceedings of the National Academy of Sciences, USA 92, 12120–12125.

Nardes V, Gültlich M, Brambilla A, Carbone ML. 1996. Disruption of the GTP-cyclohydrolase I gene in Saccharomyces cerevisiae. Biochemical and Biophysical Research Communications 218, 273–279.

Nazi KF, Sameer AS, Ganaie BA. 2014. Folate: metabolism, genes, polymorphisms and the associated diseases. Gene 533, 11–20.

Nunes AC, Kalkmann DC, Aragão FJ. 2009. Folate biofortification of lettuce by expression of a codon optimized chicken GTP cyclohydrolase I gene. Transgenic Research 18, 661–667.

Ramana Rao MV, Parameswari C, Sripiya R, Veluthambi K. 2011. Transgene stacking and marker elimination in transgenic rice by sequential Agrobacterium-mediated co-transformation with the same selectable marker gene. Plant Cell Reports 30, 1241–1252.

Ramirez Rivera NG, García-Salinas C, Aragão FJ, Díaz de la Garza RI. 2016. Metabolic engineering of folate and its precursors in Mexican common bean (Phaseolus vulgaris L.). Plant Biotechnology Journal 14, 2021–2032.

Ravanel S, Cherest H, Jibrin S, Grunwald D, Saurin-Kerjan Y, Douce R, Rébéillé F. 2001. Tetrahydrofolate biosynthesis in plants: molecular and functional characterization of dhcytofolate synthetase and three isoforms of folypolyglutamate synthetase in Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA 98, 15360–15365.

Sah R, Ravanel S, Basset G, Nichols BP, Hanson AD, Rébéillé F. 2006. Folate synthesis in plants: purification, kinetic properties, and inhibition of aminodeoxychorismate synthase. The Biochemical Journal 396, 157–162.

Scott J, Rébéillé F, Fletcher J. 2000. Folic acid and folates: the feasibility for nutritional enhancement in plant foods. Journal of the Science of Food and Agriculture 80, 795–824.

Storozhenko S, De Brouwer V, Volckaert M, Navarreto O, Blancquaert D, Zhang GF, Lambert W, Van Der Straeten D. 2007. Folate fortification of rice by metabolic engineering. Nature Biotechnology 25, 1277–1279.

Strobbe S, Van Der Straeten D. 2017. Folate biofortification in food crops. Current Opinion in Biotechnology 44, 202–211.

Tuteja N, Verma S, Sahoo RK, Raveendr S, Reddy IN. 2012. Recent advances in development of marker-free transgenic plants: regulation and biosafety concern. Journal of Biosciences 37, 167–197.

Wang K, Liu H, Du L, Ye X. 2017. Generation of marker-free transgenic hexaploid wheat via an Agrobacterium-mediated co-transformation strategy in commercial Chinese wheat varieties. Plant Biotechnology Journal 15, 614–623.

Wang K, Riaz B, Ye XG. 2018. Wheat genome editing expedited by efficient transformation techniques: progress and perspectives. The Crop Journal 6, 22–31.

Xing A, Zhang Z, Sato S, Staswick P, Clement T. 2000. The use of two T-DNA binary system to derive marker-free transgenic soybeans. In Vitro Cellular & Developmental Biology-Plant 36, 456–463.

Yin M, Zhang C, Fan K, Wang J, Wang K, Wang L, Du X, Ye X. 2018. Effects of different chemicals and treatment methods on chromosome doubling of haploid wheat plants. Scientia Agricultura Sinica 51, 811–820.

Yoo SD, Cho YH, Sheen J. 2007. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nature Protocols 2, 1565–1572.

Zhang X, Henriques R, Lin SS, Niu QW, Chua NH. 2006. Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nature Protocols 1, 641–646.